Systemic Cytokine Immunotherapy for Experimental Cytomegalovirus Retinitis in Mice With Retrovirus-Induced Immunodeficiency

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Purpose. To evaluate and compare the in vivo administration of interleukin-2 (IL-2) or interleukin-12 (IL-12) in the immunotherapy of necrotizing retinitis caused by murine cytomegalovirus (MCMV) in mice with a retrovirus-induced immunodeficiency syndrome (MAIDS).

Methods. Adult C57BL/6 mice with MAIDS of 8 weeks' duration were treated with either a single intramuscular injection of polyethylene glycol-modified human recombinant IL-2 (PEG-IL-2) or multiple intramuscular injections of murine recombinant IL-12; untreated mice with MAIDS received phosphate-buffered saline. Two days later, the left eyes of all mice were inoculated with MCMV by subretinal injection and evaluated at day 6 for intraocular MCMV titers or at day 10 for frequency of necrotizing MCMV retinitis.

Results. Infectious MCMV was significantly reduced in whole eyes of PEG-IL-2-treated mice with MAIDS (2.8 log_{10}), but not in whole eyes of IL-12-treated animals (4.4 log_{10}) when compared with whole eyes of untreated animals with MAIDS (4.5 log_{10}). Similarly, whereas eyes from ~80% of IL-12-treated and untreated mice with MAIDS showed histopathologic features consistent with classic necrotizing MCMV retinitis (full-thickness retinal necrosis associated with virus inclusions and cytomegalocytes), none (0%) of PEG-IL-2-treated animals with MAIDS showed classic MCMV retinitis. Instead, eyes from these animals showed either retinal folding or outer retinal atrophy, a pattern of histopathology similar to that observed in eyes from immunologically normal C57BL/6 mice inoculated subretinally with MCMV.

Conclusions. These results provide proof-of-principle for the hypothesis that systemic cytokine immunotherapy will reduce the frequency of CMV retinitis in a setting of retrovirus-induced immunosuppression. Because of the striking differential effects of IL-2 and IL-12 on MCMV-retinitis in mice with MAIDS, the authors conclude that cytokine immunotherapy for cytomegalovirus-induced retinitis is cytokine-specific, even for such cytokines as IL-2 and IL-12 that have T cell regulation in common.

Cytomegalovirus (CMV) retinitis is the leading cause of blindness in persons with AIDS, affecting approximately 40% of this patient population. Management of AIDS-related CMV retinitis continues to rely on traditional antiviral chemotherapy, including ganciclovir and foscarnet, despite problems associated with antiviral drugs. These include toxicities, emergence of drug-resistant virus strains, and treatment failure or breakthrough caused by the virostatic nature of these drugs. Because of the problems associated with traditional antiviral chemotherapy, we have become interested in immune-based therapies for the management of AIDS-related CMV retinitis. In particular, we hypothesize that systemic cytokine immunotherapy, either alone or in combination with traditional antiviral chemotherapy, will potentiate existing cellular immunity in HIV-1-immunosuppressed persons, thereby reducing the frequency and severity of CMV retinitis.

Unlike immune-based therapies that rely on reconstitution of the immune system by passive administration...
of immunoglobulins or immune effector cells, cytokine immunotherapy relies on restoration and preservation of function of preexisting immune effector cells. Cytokine immunotherapy is therefore well suited for HIV-1-induced immunodeficiency syndrome, during which several immune effector cell subsets, including CD8+ T cells, natural killer (NK) cells, macrophages, and neutrophils exist but fail to function because of the absence of CD4+ helper T cell activity. Because interleukin-2 (IL-2) is an important regulatory cytokine of T cell origin with potent effects on T cells, B cells, and NK cells, clinical trials of both native and recombinant IL-2 have been in progress since 1983 to assess the ability of this Th1 cytokine to restore immune function in HIV-1–infected patients. Results of clinical trials to date have been encouraging and show that intermittent courses of IL-2 coupled with antiretroviral drug therapy produces substantial and sustained increases in CD4+ T cell counts in AIDS patients with moderate immunosuppression. Results of studies in vitro have also shown that exogenous IL-2 increases the depressed NK cell activity and CMV–specific cytotoxic T cell activity of peripheral blood mononuclear cells recovered from patients with AIDS.

Clinical trials are also in progress to evaluate in HIV-1–infected patients the potential immunomodulating activity of another cytokine, interleukin-12 (IL-12). This Th1 cytokine is of monocyte, macrophage, neutrophil, and dendritic cell origin and activates NK cells and T cells. It seems particularly potent in its ability to induce production of interferon-γ, a cytokine that also helps to shape a cell-mediated immune response. Findings in animal studies have shown that IL-12 treatment not only initiates protective immune responses in mice with experimental parasitic infections, it increases splenic CD8+ T cell number in mice with experimental virus infections including murine cytomegalovirus (MCMV).

These observations provide a rationale for evaluating and comparing IL-2 and IL-12 for cytokine immunotherapy of CMV retinitis during retrovirus-induced immunodeficiency. Toward this end, we used a mouse model of experimental MCMV retinitis that occurs in mice with MAIDS, an immunodeficiency syndrome caused by a mixture of murine retroviruses. Our findings show that systemic administration of polyethylene glycol–modified recombinant IL-2 (PEG–IL-2) to mice with MAIDS before subretinal MCMV infection significantly reduced titters of ocular virus as well as frequency of classic MCMV necrotizing retinitis. In comparison, IL-12 showed no significant therapeutic effect.

MATERIALS AND METHODS

Animals
Adult female C57BL/6 euthymic mice (Taconic Farms, Germantown, NY) were used for all retinitis experiments. Mice were allowed unrestricted access to food and water and maintained in alternating 12-hour light-dark cycles. All animal procedures were performed in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Viruses
Stocks of the Smith strain of MCMV were prepared in salivary glands of BALB/c mice, titered on monolayers of mouse embryo fibroblasts, and stored in liquid N2. A fresh aliquot of MCMV stock was thawed and used for a single experiment. Stocks of the murine retrovirus mixture (LP–BM5 MuLV) were prepared using SC-1–MuLV LP–MB5 cells obtained through the AIDS Research and Reference Reagent Program National Institutes of Health (Bethesda, MD).

Cytokines
Polyethylene glycol–modified human recombinant IL-2 (PEG–IL-2; 4 × 10^6 IU/mg) was a kind gift from Chiron Corporation, Emeryville, CA. A new vial of PEG–IL-2 was reconstituted in sterile distilled H2O for each experiment immediately before use and administered as a single intramuscular injection (thigh muscle of left or right leg) at a dose of 23.5 μg (1 mg/kg per mouse) as suggested by previous in vivo studies (Giedlin M, unpublished data, 1994). The biologic activity of this PEG–IL-2 preparation was confirmed using an IL-2–dependent mouse T cell line that undergoes apoptotic cell death in the absence of IL-2 (data not shown).

Murine recombinant IL-12 (Genetics Institute, Cambridge, MA) (2.75 × 10^6 IU/0.5 mg) was kindly provided by Dr. Bruce Ksander (Schepens Eye Institute, Boston, MA). The biologic activity of this IL-12 preparation was confirmed in an unrelated mouse study. Pilot experiments determined a dosage of 200 μg to be the lowest nontoxic dose of IL-12 tolerated by animals with MAIDS after multiple intramuscular injections at days −2, 0, and 2 relative to subretinal MCMV injection. The IL-12 preparation was determined by the manufacturer to be endotoxin free.

Induction of MAIDS
MAIDS was induced in C57BL/6 mice by intraperitoneal injection of 1 ml LP–BM5 MuLV preparation containing approximately 5 × 10^5 to 5 × 10^6 infectious murine retroviruses. Mice infected for 8 weeks with LP–BM5 MuLV displayed clinical features consistent with the development of MAIDS including massive lymphadenopathy and splenomegaly.

Subretinal Infection With Murine Cytomegalovirus
Approximately 1 × 10^4 plaque-forming units of MCMV contained within a volume of 2 μl was injected subreti-
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nally into the left eyes of mice, using a modified suprachiliary injection procedure.16

Recovery of Infectious Murine Cytomegalovirus

Immediately after removal from euthanized animals, whole eyes were homogenized individually using aseptic conditions in 1 ml of cold DMEM (Dulbecco’s modified Eagle’s tissue culture medium containing 10% fetal bovine serum) and clarified by centrifugation. Tenfold dilutions of the resulting supernatants were titrated in duplicate onto monolayers of mouse embryo fibroblast cells, allowed to adsorb for 1 hour at 37°C, overlaid with methylcellulose containing DMEM, and incubated for 5 to 6 days at 37°C in a humidified CO2 atmosphere. Individual plaques were counted using an inverted microscope. Results are reported as PFU per milliliter per eye and represent the mean titers of three to five eyes per group.

Histopathologic Preparation and Evaluation

At 10 days after subretinal MCMV injection, eyes were carefully removed from euthanized animals, fixed in 10% buffered formalin, paraffin-embedded, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. At least six histopathologic sections representing different regions of the retina were examined and scored for frequency of MCMV necrotizing retinitis using a histopathologic grading criteria described previously.12 Posterior segments of eyes that were positive for MCMV retinitis exhibited full-thickness retinal necrosis associated with cytomegalic cells (cytomegalocytes).

RESULTS

Effect of Interleukin-2 or Interleukin-12 Treatment on Ocular Murine Cytomegalovirus Titers

We have shown previously that whole eyes of mice with MAIDS contain significantly higher amounts of infectious virus after subretinal inoculation with MCMV when compared with amounts of virus in eyes from immunocompetent control animals.12 Experiments were therefore performed to evaluate and compare PEG–IL-2 and IL-12 for their ability to reduce intraocular titers of MCMV in mice with MAIDS. PEG–IL-2 was administered by a single intramuscular injection 2 days before subretinal inoculation with MCMV, whereas IL-12 was administered by multiple intramuscular injections at days −2, 0, and 2, relative to subretinal injection of MCMV. Untreated control mice received intramuscular injections of sterile distilled H2O only (Fig. 1). In agreement with previous work,12 MCMV–inoculated eyes of untreated animals with MAIDS consistently showed high amounts of infectious MCMV (4.5 log10) when compared with that found in MCMV–inoculated eyes of normal immunocompetent mice (2.5 log10). In comparison, MCMV–inoculated eyes of IL-12–treated mice with MAIDS showed amounts of infectious MCMV (4.4 log10) equivalent to that found in untreated animals with MAIDS. In sharp contrast, however, PEG–IL-2–treated mice with MAIDS showed a dramatic decrease in ocular titers of MCMV (2.8 log10), which was significant when compared with titers of MCMV in control eyes from untreated animals with MAIDS (P < 0.001). Moreover, the amount of infectious MCMV observed in eyes of PEG–IL-2–treated animals with MAIDS was similar to that observed in eyes of normal immunocompetent C57BL/6 mice subjected to subretinal inoculation with MCMV. The reproducibility of systemic PEG–IL-2 treatment to reduce the amount of infectious virus in MCMV–inoculated eyes of animals with MAIDS was confirmed in additional experiments in which PEG–IL-2 was administered intramuscularly either 1 day or 3 days before subretinal inoculation with MCMV (Fig. 1). Taken together, these data suggest that systemic injection of PEG–IL-2, but not IL-12, results in decreased intraocular MCMV replication when administered to mice with retrovirus-induced immunosuppression before subretinal inoculation with MCMV.
TABLE 1. Frequency of Murine Cytomegalovirus Necrotizing Retinitis

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<tr>
<th>Group</th>
<th>Frequency (%) of Necrotizing Retinitis (retinitis/total)</th>
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<tbody>
<tr>
<td>Immunocompetent</td>
<td>0 (0/6)*</td>
</tr>
<tr>
<td>Untreated MAIDS</td>
<td>80 (8/10)</td>
</tr>
<tr>
<td>IL-12-treated MAIDS</td>
<td>83 (10/12)</td>
</tr>
<tr>
<td>IL-2-treated MAIDS</td>
<td>0 (0/15)*</td>
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MAIDS = murine acquired immunodeficiency syndrome; IL = interleukin.
* Significantly different from untreated MAIDS control group, \( P \leq 0.001 \) (chi-square).

Effect of Interleukin-2 or Interleukin-12 Treatment on Frequency of Murine Cytomegalovirus Necrotizing Retinitis

Mice with MAIDS show increased susceptibility to MCMV necrotizing retinitis after subretinal inoculation with MCMV when compared with susceptibility in immunocompetent control mice. Because of the dramatic decrease in intraocular titers of MCMV observed in animals with MAIDS receiving systemic PEG-IL-2 before subretinal inoculation with MCMV, it was of interest to examine eyes from these mice and compare them with eyes from IL-12-treated mice for frequency of MCMV necrotizing retinitis. Groups of mice with MAIDS were treated intramuscularly with either PEG-IL-2 or IL-12 2 days before subretinal inoculation with MCMV; control mice received distilled H2O intramuscularly. Histopathologic analysis of eyes recovered from control and IL-12-treated mice with MAIDS at 10 days after subretinal inoculation with MCMV revealed classic MCMV necrotizing retinitis in 80% (8 of 10) and 83% (10 of 12) of the animals, respectively (Table 1). Histopathologic features of eyes from control (Fig. 2) or IL-12-treated (Fig. 3) animals with MAIDS included full-thickness retinal necrosis, retinal cells and retinal pigment epithelium with prominent virus-induced inclusions, and foci of cytomegalocytes. In sharp contrast, none (0%) of the eyes recovered from PEG-IL-2-treated mice with MAIDS showed histopathologic features of classic MCMV necrotizing retinitis. Instead, sections of eyes from these animals showed either retinal folding or outer retinal atrophy (Figs. 4A, 4B), a pattern of histopathology similar to that observed in sections of eyes from immunologically normal C57BL/6 mice inoculated subretinally with MCMV (Fig. 5). Thus, systemic PEG-IL-2 treatment of mice with MAIDS before subretinal inoculation with MCMV not only sharply reduced intraocular MCMV replication to levels found in immunocompetent mice, but also decreased the development of classic MCMV necrotizing retinitis, findings not observed in IL-12-treated animals with MAIDS.

DISCUSSION

We have found, using an experimental animal model of CMV retinitis, that systemic treatment of retrovirus-immunosuppressed mice with PEG-IL-2 before subretinal inoculation with MCMV leads to reduced intraocular MCMV replication and a decrease in the frequency of classic MCMV necrotizing retinitis. This finding provides proof-of-principle for our hypothesis that cytokine immunotherapy will reduce the frequency of CMV retinitis in a setting of retrovirus-induced immunosuppression.

Our additional finding that IL-12 treatment of mice with MAIDS had no effect on ocular MCMV dis-
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Figure 4. Photomicrographs of the retinas of polyethylene glycol–interleukin-2–treated C57BL/6 mice with retrovirus-induced immunodeficiency (MAIDS) 10 days after subretinal inoculation with murine cytomegalovirus. There is (A) prominent retinal folding and (B) outer retinal atrophy (arrows). Hematoxylin and eosin; original magnification, ×200.

Figure 5. Photomicrograph of the retina of an untreated immunocompetent mouse 10 days after subretinal inoculation with murine cytomegalovirus. There is prominent retinal folding. Hematoxylin and eosin; original magnification, ×200.

The polyethylene glycol modification of the human recombinant IL-2 used in this study may have contributed to its overall therapeutic efficacy. Covalent attachment of PEG to IL-2 produces a hydrophilic molecule with an apparent molecular weight of approximately 160K that is completely soluble at any pH.19-21 These characteristics result in an extended in vivo circulation half-life that is approximately 4 times greater than that of unmodified recombinant IL-2. Nevertheless, the striking differential effects on experimental CMV retinitis during MAIDS observed after a single PEG–IL-2 administration of PEG–IL-2 when compared with multiple administrations of IL-12 suggest cytokine-specific antiviral pathways.

The precise mechanism whereby IL-2 treatment protects against MCMV retinitis in mice with MAIDS has yet to be determined. We suspect that administration of IL-2 to mice with MAIDS activated existing CD8+ T cells, NK cells, or cells associated with a nonspecific inflammatory response (that is, macrophages and neutrophils) thereby leading to clearance of virus from ocular tissues and subsequent prevention of necrotizing retinitis. Roles for CD8+ T cells and NK cells in this process have been suggested by depletion studies in immunocompetent mice.22,23 In addition, LAK cells could play a role in protection during IL-2 immunotherapy. Lymphokine-activated killer cells are thought to be similar to NK cells in that they lyse target cells in a non–MHC–restricted manner, but may possess mechanisms for cytotoxicity that are regulated differently from those in NK cells.24-26

The timing of IL-2 administration relative to virus infection may also be important for successful IL-2 immunotherapy. Although significant therapeutic efficacy was consistently observed when IL-2 was administered at 1, 2, or 3 days before subretinal inoculation with MCMV, preliminary experiments suggest that therapeutic efficacy is lost in animals with MAIDS when IL-2 is administered at the same dosage 1 day after subretinal inoculation with MCMV. This preliminary finding could merely reflect an imperfection of the animal model—an inability of IL-2 immunotherapy to cope with an overwhelming dose of virus injected subretinally several hours before the cytokine...
is administered. Alternatively, however, IL-2 immuno-
therapy may ultimately prove ineffective in the treat-
ment of preexisting CMV retinitis during AIDS.

In addition to treatment of preexisting CMV retini-
tis, cytokine immunotherapy could conceivably be used
prophylactically to reduce or prevent spread of virus to
the eye and thereby delay or prevent onset of retinitis
during AIDS. Intraperitoneal inoculation of immuno-
competent mice with a genetically engineered strain of
MCMV that produces β-galactosidase during replica-
tion has shown that virus disseminates through mono-
cytes–macrophages to major target organs and tissues
of the animal, including spleen, liver, lungs, and sali-
vary glands.27 The tracer virus also travels to the eye of
immunosuppressed mice (including mice with MAIDS)
after systemic inoculation28 (Dix RD, Cousins SW, un-
published data, 1995). However, despite evidence for
infection of several ocular tissues (iris, ciliary body, reti-
nal pigment epithelium, and choroid) with this virus, the
neuroretinal retina is spared and retinitis fails to
develop, suggesting a need for as yet undefined cofac-
tor(s) required to convert the occult subclinical infec-
tion to an overt clinical retinitis.29 We have not yet
explored the potential prophylactic value of cytokine
immunotherapy in preventing the spread of tracer virus
to the eye during systemic MCMV infection of mice
with MAIDS. Findings in preliminary work, however,
have shown that administration of IL-2 during MAIDS
reduced the spread of infectious virus to the spleen
after intraperitoneal MCMV inoculation. This prelimi-
nary finding is consistent with the work of Reddehase
et al30 who reported that human recombinant IL-2–
responsive effector lymphocytes administered to irradi-
ated immunocompromised BALB/c mice showed pro-
phylactic value in controlling MCMV disease during
systemic virus infection.

We envision cytokine immunotherapy as an ad-
just to traditional antiviral chemotherapy for optimal
management of AIDS-related CMV retinitis. Work is
therefore presently oriented toward assessing the po-
tential synergistic value of combination IL-2 and sys-
temic ganciclovir therapy in reducing ocular titer of
MCMV or frequency of necrotizing retinitis during
MAIDS.

Key Words
cytomegalovirus retinitis, interleukin-2, interleukin-12, mu-
rine cytomegalovirus, murine AIDS (MAIDS)

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